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**FOR THE RECORD**

# Evolutionary links between FliH/YscL-like proteins from bacterial type III secretion systems and second-stalk components of the $F_oF_1$ and vacuolar ATPases

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## Abstract

Bacterial type III secretion drives flagellar biosynthesis and mediates bacterial–eukaryotic interactions. Type III secretion is driven by an ATPase that is homologous to the catalytic subunits of proton-translocating ATPases, such as the  $F_oF_1$  ATPase. Here we use PSI-BLAST searches to show that some noncatalytic components are also conserved between type III secretion systems and proton-translocating ATPases. In particular, we show that the FliH/YscL-like proteins and the E subunits of vacuolar ATPases represent fusions of domains homologous to second-stalk components of the  $F_oF_1$  ATPase (the b and  $\delta$  subunits).

**Keywords:**  $F_oF_1$  ATPase; vacuolar ATPase; bacterial flagellum; FliH; YscL; type III secretion; sequence homology; evolution

**Supplemental material:** see [www.proteinscience.org](http://www.proteinscience.org)

The bacterial flagellum, the chief organelle of motility in bacteria, is a nano-propeller driven by a rotary motor, fueled by a proton-potential gradient (Macnab 2004; Minamino and Namba 2004). Assembly of the flagellar filament depends on a dedicated flagellar type III secretion system (F-T3SS), energized by an ATPase (Vogler et al. 1991). A closely related ATPase also drives protein secretion through nonflagellar type III secretion systems (NF-T3SSs), which mediate translocation of bacterial effector proteins into eukaryotic cells (Eichelberg et al. 1994) (Fig. 1A).

It has been known for some time that the ATPases from F-T3SSs and NF-T3SSs are homologous to the

catalytic subunit of the  $F_oF_1$  “F-type” ATPase and other related proton-driven archaeal and vacuolar “V-type” ATPases (Albertini et al. 1991; Vogler et al. 1991; Gogarten et al. 1992; Eichelberg et al. 1994) (Fig. 1A). The F-type ATP synthases are ubiquitous motor complexes that couple the proton gradient across the cellular membrane to the synthesis of ATP by rotary catalysis. V-ATPases occur in archaea and some bacteria and are present on vacuoles, lysosomes, endosomes, secretory vesicles, and Golgi of all eukaryotic cells, where they maintain the acidic pH in these organelles. In some specialized cells, they are also located on the plasma membrane, mediating urine acidification, bone resorption, and cytosolic pH regulation, among other things.

Recently, while engaged in homology searches aimed at surveying the phylogenetic distribution of T3SS proteins, we noticed sequence similarity between another flagellar component FliH (homologous to YscL-like

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**B** Ypest\_YscL 46 **LISA**AKQDAEK**IL**ADAQEVY--QOKQLGWQAGMD**EART**-----LQAT**LI**HE-----  
Bjapo\_NolV 35 **IL**AAAAERH**LQ**RV**R**WAR**L**AYE--RERAAQ**G**RAG**E**AKAG**E**-----EMAR**LI**AQ-----  
Psyri\_HrpE 34 **IL**ADARQ**AT**Q**IL**AL**E**Q**KA**E-------HL**Q**Q**KL**AG**F**WE-----NAN**FL**GE-----  
Stsyph\_FliH 39 **P**EL**T**AB**Q**Q**L**EQ**L**A**L**Q**L**K**I**QA**H**(5)AG**L**AEGRQ**K**HA**Q**GY**E**(8)**G**QA**Q**A**Q**T**Q**QA**P**I**H**ARM**Q**Q(4)  
Ypest\_FliH 49 **M**E**A**T**A**EVQ**Q**LV**L**Q**N**LE**A**E(5)**Q**GF**A**K**G**L**Q**EG**L**DK**G**Y**T**(8)**A**L**A**D**A**EQ**Q**L**A**PT**A**H**V**Q**V**(4)  
Pysyri\_FliH 54 **P**L**D**EV**Q**PL**T**LE**L**ES**I**RQ**E**AW(2)**G**F**A**T**E**Q**K**E**G**F**L**ST**Q**L**K**-----VRQ**E**AEVV**L**AK**V**AS**L**EQ(4)  
Mjann\_VatE 2 **K**LM**G**VD**K**IK**S**IL**D**DA**K**AE**A**N(1)**I**SE**A**EA**E**KA**F**IL**E**KA**E**(8)**L**IK**G**KE**G**AE**M**T**S**R**I**IS(1)  
Chydr\_AtpF 45 **L**E**K**AA**K**AQ**E**AE**R**IK**A**E**Y**D**G**M-----IA**K**ARE**A**RE**R**II**A**QA**K**(8)**I**IA**T**AQ**R**EA**S**LL**A**DA**K**AT  
Ecoli\_AtpF 44 **L**AS**A**ER**A**HK**D**LD**L**AK**A**S**A**T**D**Q-----L**K**KA**K**AE**A**Q**V**II**E**QA**K**(8)**A**KA**E**AE**R**Q**T**K**I**VA**Q**AE-----  
Efecai\_AtpF 55 **L**DS**A**EQ**S**R**I**NS**A**K**M**EQ**R**ER**Q**Q-----LL**A**SR**S**DA**D**DI**I**KN**A**KE(8)**L**IK**D**AQ**E**EA**R**L**K**SA**Q**AD-----

Ypest_YscL	TQLQCQQFYRHFVEQQMSEVVLAVRKILNDYDQVA--MTLQVVR	135
Bjapo_NoIV	ATCELAQRKAVLEREPQLVFVEISRDLLGAFDPSGK--MLVQCVR	124
Psyri_HrpE	LQVQREALQEAMTAVEEELSESLRHLLDDTTLAE--RARALRNA	129
Styph_FliH	FQNTLDALDSVTSASRLMQALEAAKQHVIGQTPAVDNSALIKQIQ	158
Ypest_FliH	FQNTLDALDSVTSASRLVQIALAAKQIQVIGQTPACDGTALLAQIQ	161
Psyri_FliH	LLAPIAEQDQTIEKAVIHLVEHIAKQVIGQRELVTDSGQIASVL	168
Mjann_AtpE	AKLEAKKKLLEAKEETIMATNKLEELVKLPBPQ--EYDKLIKLI	113
Chydr_AtpF	IAQEKEKALRELRQETGNLAVLAGKILNRAVTL--DHQKLVDEF	154
Ecoli_AtpF	IEAERKKRARELRQETGVAILLVAEAKKILNRSVDEA--ANSIDV	153
Efaec_AtpF	ITVERDSALNSVKDDVAELSLSQIAEKILNKELSPSE--MHESL	164

**C**

Ypest_YscL	146	VRVNP	DQAGATREQ	IAKVHK	(4)	ISYLEV	TADARLDQ	GGCILE	TEVGI	IDAS	199												
Bjapo_No1V	135	LHVSP	LKADLLASE	FKDYDG	(3)	RPKLR	IEADP	ALSADQ	CVLW	SEFGN	VDLGL	187											
Psyri_HrpE	130	LSVHP	QIADAAE	VLDSRF	--	SEHWQ	LKRDA	TIASD	SLRSD	ANGAF	DI	179											
Styph_FliH	170	LRVHP	DDLQRVE	EMLGATLS	--	LHGWR	LRG	PTLH	HGGCK	VSAD	EGD	LDASV	219										
Ypest_FliH	180	LRVNP	DDLAI	VEQRL	GSTLS	--	LHGWR	LRG	DSQI	HAGGCK	VS	AEGD	LDASL	229									
Psyri_FliH	173	FIFNP	QDFLL	VKAM	--	RERH	--	EEAWK	I	VED	EDLL	PGG	CRITE	HSIR	IDAS	220							
Mjann_VatE	133	ELID	DDSTL	WNLEKE	EVEN	NATK	--	KVTVL	KCG	FPV	DI	AGGCI	ETAD	GLK	SLDN	184							
Avari_AtpD	117	VALTE	DQQA	VKEK	VLAL	TK	(2)	QVELAT	KV	DS	DL	-	IGG	VII	KVGS	QV	IDS	168					
Teryt_AtpD	116	VELN	ENQK	NTIR	RVK	QMS	(2)	EVEI	ET	KV	DS	EL	-	LG	GV	II	KIGS	QI	IDS	168			
Pmari_AtpD	115	SALNE	DQ	QSE	LE	RRV	K	SI	AG	(2)	NLEI	DL	KV	DS	EL	-	LG	GV	II	KIGS	QI	IDS	168
Ecoli_AtpD	113	AALSE	QOLAK	IS	AAME	KRL	(2)	KVKL	NC	KI	DS	KV	-	MAG	VI	IR	AG	DM	IV	DS	164		

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(Jackson and Plano 2000; Matzke 2003; Lane et al. 2005).

We therefore sought to perform a more sophisticated bioinformatics analysis, in the hope of strengthening and extending the claims for homology between type III secretion systems and proton-translocating membrane-associated ATPases. We chose to use the homology search tool PSI-BLAST for this purpose, because it is highly sensitive and also provides a statistical measure of the significance of any finding through the “expect value” (or “e value”) (Altschul et al. 1997).

## Results

A PSI-BLAST search was performed with YscL, a FliH-like protein from the Ysc-Yop NF-T3SS from *Yersinia*. Within the first iteration, unequivocal (e value < 0.005), near-full-length homology was revealed to 73 proteins, including several FliH homologs and a variety of proteins from NF-T3SSs (obvious YscL homologs, plus proteins annotated as NolV in the rhizobial systems and HrpB5 in *Xanthomonas*).

The second iteration revealed significant hits to 291 proteins, including many more FliH proteins and NF-T3SS proteins (e.g., proteins annotated as HrpF in *Ralstonia* and *Acidovorax*, HrpE in *Pseudomonas syringae*). However, the crucial finding in this iteration was that YscL showed significant similarity to the b subunits of F-type ATPases (AtpF proteins) from seven bacterial genera (spanning three phyla: green-sulfur bacteria, Gram-positives, and chloroflexi) and to the E subunits of V-type ATPases from spirochetes and from three archaeal genera.

The b subunit yielding the most impressive e value was from green sulfur bacterium *Prosthecochloris aestuarii* (e value = 1e-05; identities = 20/111, or 18%), while the

best-scoring E subunit came from *Methanocaldococcus janneschii* (e value = 9e-06; identities = 35/175, or 20%). When a third iteration was run, many FliH homologs (including FliB from *Caulobacter crescentus*) and many more b and E subunits were returned with significance; however, some low complexity proteins contaminated the matrix, rendering further iterations unhelpful (Fig. 1B).

To confirm the homology, we performed a PSI-BLAST search with the *Methanocaldococcus janneschii* E subunit (Swissprot entry VATE\_METJA). Within three iterations, significant similarity was reported to YscL and numerous other YscL/FliH-like proteins, and to numerous V-ATPase E subunits from archaea and eukaryotes (including VATE\_HUMAN) and numerous F-ATPase b subunits from bacteria.

Scrutiny of the alignments revealed that the similarity between the E subunits from V-type ATPases and the YscL/FliH-like proteins encompassed nearly the full length of both kinds of protein. However, although the alignments between YscL and F-ATPase b subunits encompassed almost all of the b subunit sequence, they covered only the N-terminal region of YscL. We therefore repeated automated PSI-BLAST searches with the C-terminal region of YscL (residues 115–223 in YSCL\_YEREN), adjusting the parameters to modulate sensitivity (the “composition-based statistics” option was turned off).

After just one iteration, PSI-BLAST reported credible similarity between the C terminus of YscL and the  $\delta$  subunit from the F-type ATPase from the cyanobacterium *Trichodesmium erythraeum* (expect = 0.086; identities = 29/106, or 27%).  $\delta$  subunits from two other cyanobacteria also featured in the list of hits: *Prochlorococcus marinus* (expect = 4.7; identities = 22/100, or 22%) and *Anabaena variabilis* (expect = 8.1; identities = 26/104, or 25%). Curiously, with the second iteration, the cyanobacterial proteins disappeared from the results list, but four new  $\delta$

**Figure 1.** (A) Diagrammatic depiction of proton-translocating and type III secretion ATPase complexes, highlighting homologous catalytic and noncatalytic components and interactions. (B,C) Multiple alignments of representative NF-T3SS, F-T3SS, F-type, and V-type ATPases. (B) N-proximal domains of NF-T3SS, F-T3SS, and V-type ATPases aligned with F-type B subunit proteins. (C) C-terminal domains of NF-T3SS, F-T3SS, and V-type ATPases aligned with C-terminal domains of F-type D subunit proteins. Alignments were generated using T-coffee ([http://igs-server.cnrs-mrs.fr/~cnotred/Projects\\_home\\_page/t\\_coffee\\_home\\_page.html](http://igs-server.cnrs-mrs.fr/~cnotred/Projects_home_page/t_coffee_home_page.html)) and a set of 32 representative sequences derived from the original YscL psiBLAST. For clarity, the final alignments contain only 3 NF-T3SS ATPases, 3 F-T3SS ATPases, 1 V-type ATPase, 3 F-type B subunits, and 4 F-type D subunits. Alignments were colored using CHROMA (<http://www.lg.ndirect.co.uk/chroma/>) with an 80% consensus threshold: aromatic (FHXY, blue lettering on a dark yellow background), big (EFHIKLMQRWY, blue on light yellow), hydrophobic (ACFGHILMTVWY, black on dark yellow), aliphatic (ILV, gray on dark yellow), polar (CDEHKNNQRST, blue on white), small (ACDGNPSTV, dark green on white), tiny (AGS, light green on white), charged (DEKR, pink on white), and negatively charged (DE, red on white). Organism names are abbreviated as Ypest (*Yersinia pestis*), Bjapo (*Bradyrhizobium japonicum*), Psyri (*Pseudomonas syringae*), Styph (*Salmonella typhimurium*), Mjann (*Methanococcus janneschii*), Chydro (*Carboxydotherrmus hydrogenoformans*), Ecoli (*Escherichia coli*), Efaec (*Enterococcus faecium*), Pmari (*Prochlorococcus marinus*), Avari (*Anabaena variabilis*), and Teryt (*Trichodesmium erythraeum*), respectively. Protein names are as stated in the respective GenBank record, except where no name is available (i.e., Efaec\_AtpF, Pmari\_AtpF, and Teryt\_AtpF are EfaeDRAFT\_0503, PMN2A\_0982, and TeryDRAFT\_3685, respectively). GenBank accession numbers (in alignment order) are 10955581, 27376926, 28868594, 16765309, 16122081, 28869164, 15668393, 78042703, 16131604, Efae021782, 75908826, 71674228, 72382820, 114580.

subunits from two additional bacterial phyla (firmicutes, proteobacteria) and the C terminus of one V-type E subunit joined the list with levels of identity ranging from 19% to 26%, albeit with unimpressive *e* values (1.5–8.5). Frustratingly, no  $\delta$  subunit ever reached the default threshold for inclusion in the PSI-BLAST matrix (0.005), no matter how many iterations were run.

A PSI-BLAST search starting with the  $\delta$  subunit from *Trichodesmium erythraeum* reported, within a single iteration, unequivocal homology with many other  $\delta$  subunits from bacteria (including AtpH from *E. coli*), and to their equivalents from mitochondria (the oligomycin sensitivity conferral protein, or OSCP). Importantly, the search also reported similarity to the C-terminal half of YscL (expect = 0.14; identities = 29/106, or 27%), confirming the reciprocal nature of the sequence similarity. However, mirroring earlier results, no YscL/FliH-like protein ever crossed the PSI-BLAST default inclusion threshold. When AtpH from *E. coli* was employed as query sequence, within two iterations, PSI-BLAST uncovered similarity with the C termini of FliH proteins: from the Gram-positive *Moorella thermoacetica* (expect = 2.3; identities = 17/94, or 18%) and the spirochete *Leptospira interrogans* (expect = 3.7, identities = 16/99, or 16%) (Fig. 1C).

## Discussion

The sequence analyses reported here provide solid support for the notion that the N-terminal region of the FliH/YscL-like proteins is homologous to the b subunit of the  $F_1 F_0$  ATPase and that there is full-length homology between these proteins and the V-type E subunit. The evidence from sequence homology is backed up by similarities in structure and function. All three groups of proteins adopt extended nonglobular structures (through the “tether domain” in the b subunit). Furthermore all three dimerize, which in the case of FliH and the b subunit is known to occur through interactions involving a domain C-terminal to the extended domain (Dunn et al. 2000; Gonzalez-Pedrajo et al. 2002; Minamino et al. 2002; Fethiere et al. 2004).

The precise nature of the homodimeric interaction between the two subunits in the b subunit is unclear: It has been suggested based on the structure of an isolated dimerization domain that it might involve a unique right-handed coiled-coil interaction (Del Rizzo et al. 2002). Whatever the mechanism, the assertion of homology between the b subunit and the FliH/YscL-like proteins implies that FliH will employ a similar dimerization strategy and that there will be obvious similarities in structure between FliH and the b subunit. Similar arguments apply to structural and functional features of the N-terminal region of the poorly understood E subunit

from the V-type ATPases (Fethiere et al. 2004; Owegi et al. 2005).

Curiously, unlike the b subunit (Dmitriev et al. 1999), neither the FliH/YscL-like proteins nor the E subunits possess an N-terminal trans-membrane domain. This suggests that other proteins must mediate membrane association in this system. In the flagellar system from *Salmonella*, the N terminus of FliH mediates interactions with FliJ, which in turn interacts with the soluble cytoplasmic domain of the largest membrane component of the export apparatus, FlhA (Gonzalez-Pedrajo et al. 2002; Fraser et al. 2003). In the flagellar system of *Helicobacter pylori*, which lacks a FliJ homolog, two other additional proteins interact with FliH (HP0958, HP0406) and probably mediate interactions with the rest of the secretion apparatus (Lane et al. 2005; Ryan et al. 2005). In addition to its interaction with the ATPase, YscN, YscL is known to interact with YscQ, a homolog of the C-ring component FliN (Jackson and Plano 2000).

The F-type b subunit and the V-type E subunits have been shown experimentally to be generally permissive of point mutations and small insertions and deletions that lengthen or shorten the stalk (Sorgen et al. 1999; Grabar and Cain 2003; Bhatt et al. 2005; Owegi et al. 2005). Alignments of distantly related members of the FliH family, which often feature indels, suggest that this is also true of these flagellar proteins: a prediction that can be easily tested experimentally.

The sequence similarities reported here between the C termini of YscL/FliH-like proteins and the ATPase  $\delta$  subunits suggest that there is homology between these two families of proteins. Although backed by rather unimpressive PSI-BLAST *e* value statistics, several other lines of evidence reinforce this claim. Firstly, both kinds of protein bind to amphipathic helices at the N termini of homologous ATPases (Lane et al. 2005). This observation recently led Lane and colleagues to propose, quite independently of the sequence data presented here, that the FliH–FliI and the  $F_1 \alpha$ – $\delta$  subunit interactions were “analogous.” The PSI-BLAST similarities presented here allow us to go one further and assert that the relationship is instead “homologous.”

Secondly, the idea that a single protein in the type III secretion systems (and in the V-type ATPases) corresponds to two proteins in the F-type ATPase is backed up by the fact that the b and the  $\delta$  subunits interact to form the second stalk of the ATPase and are encoded by adjacent genes in bacteria (Wilkins and Capaldi 1998).

As before, the assertion of homology allows us to make new predictions, for example, that the structures of the C-terminal domains of the FliH/YscL-like proteins will, at least in part, resemble the  $\delta$  subunit, which contains an N-terminal  $\alpha$ -helical bundle and an uncharacterized C-terminal  $\alpha$ – $\beta$  fold (Wilkins et al. 1997).

Similarly we can predict that homologous protein–protein interactions in the proton-translocating ATPases and in the flagellar and nonflagellar type III secretion systems (e.g., OrgB–InvC, Spa47–MxiN) will involve similar intermolecular interfaces.

In addition to strengthening the links between proton-translocating ATPases and type III secretion, our PSI-BLAST searches bring many cryptic nonflagellar type III secretion proteins—annotated with confusingly variable nomenclatures (HrpB5, HrpE, HrpF, NolV)—into the FliH/YscL family. Curiously, some of the better characterized members of the family (e.g., OrgB from Spi-1, Spa47 from *Shigella*, Orf5 from the LEE), are absent from searches starting with YscL but can be joined to the family by searches with other query sequences (data not shown). Interestingly, a puzzling flagellar protein of uncertain function, FlbE from *Caulobacter crescentus* (Muir and Gober 2001), also joins the FliH/YscL family through our searches. In all of these cases one can make a solid prediction that these FliH/YscL-like proteins will interact with the ATPase from their host secretion system.

The newfound homologies between type III secretion systems and proton-translocating ATPases raise new problems. A key point is that previous experimental evidence suggests that the interaction between the b and the  $\delta$  subunits is mediated by the C termini of each protein (Wilkins et al. 1997). Yet our conclusion that homologous sequences are fused into a single protein in the type III secretion systems seems to imply that it should be the N terminus of the  $\delta$  subunit that interacts with the C terminus of the b subunit.

One solution to this conundrum might be that some of the sequence corresponding to the N-terminal domain of the  $\delta$  subunit might be missing in the FliH/YscL-like proteins—the PSI-BLAST alignments do not include the first 80 or so residues of the  $\delta$  subunit. Furthermore, an additional piece of evidence to support our thesis comes from the fact that these two subunits can fuse C terminus to N terminus into one protein, at least on an evolutionary timescale, without loss of function—the mycobacterial F-type ATPase gene cluster contains a gene (Rv1307) that encodes a fusion of the b and the  $\delta$  subunits. Interestingly, this gene cluster also encodes a second homolog of the b subunit (Rv1306), suggesting that in this bacterium the usual homodimeric b–b interaction is replaced by a heterodimeric interaction that maintains the b<sub>2</sub>– $\delta$ <sub>1</sub> stoichiometry. We suspect that a similar arrangement occurs in the V-type systems, where the E–G complex represents a b<sub>2</sub>– $\delta$ <sub>1</sub> stoichiometry (the G subunit is a second homolog of the F-type b subunit in this system) (Fethiere et al. 2004).

A deeper problem is that we still have little idea what functions the FliH/YscL-like proteins contribute to type

III secretion. FliH inhibits the ATPase activity of FliI in vitro, which has led to the suggestion that in vivo it might prevent futile ATP consumption in the absence of protein secretion (Minamino and MacNab 2000). Equally puzzling is why the interaction between FliH-like proteins and FliI-like proteins has been conserved over such long periods of evolutionary time, when flagellar motility is still possible, albeit less efficient, in the complete absence of FliH (Minamino et al. 2003). However, it is worth noting that in several nonflagellar T3SSs, YscL homologs, such as HrpB5, Orf5/EscL are known to be required for protein secretion (Rossier et al. 2000; Deng et al. 2004).

The b and the  $\delta$  subunits form a second stalk in the F-type ATPases that is thought to act as a stator preventing rotation of the catalytic subunits relative to the torque-generating  $\gamma$  subunit (Wilkins and Capaldi 1998). This raises the intriguing idea that the FliI-like ATPases in type III secretion systems might induce torque as they deliver proteins to the secretion apparatus, and that FliH-like proteins might thus also act as stators (Matzke 2003; Lane et al. 2005). If so, this might in turn suggest that an ATP-driven rotary motor could have powered the protoflagellum and acted as a precursor to today's more sophisticated proton-driven flagellar motor (Matzke 2003).

The discovery of extensive homology between type III secretion systems and membrane-associated proton-translocating ATPases allows us to glimpse an association between catalytic and noncatalytic subunits that predates the divergence of these systems from a primordial ATPase, which itself probably predates the last universal common ancestor. The power of evolutionary thinking to highlight parallels between such important, but superficially unrelated, molecular systems provides yet another example, to quote Darwin, of the “grandeur in this view of life” (Darwin 1859).

## Methods

PSI-BLAST searches were initiated on the NCBI's BLAST server (<http://www.ncbi.nlm.nih.gov/BLAST/>) on November 5, 2005, using query sequences as detailed in the text and under default conditions unless stated otherwise. In the first search, the first 11 residues of the Swissprot entry YSCL\_YEREN were removed to leave a 212-residue query sequence, as TBLASTN searches against the genomes of bacteria with related type III secretion systems (*Pseudomonas aeruginosa*, *Photobacterium luminescens*) suggested that the true YscL coding sequence started at residue 12 of the database sequence (data not shown).

## Electronic supplemental material

PSI-BLAST results are available as Supplemental Material.

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